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Expression and Antigenicity of Human Herpesvirus 8 Encoded ORF59 Protein in AIDS-Associated Kaposi's Sarcoma

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Human herpesvirus 8 (HHV-8, Kaposi's sarcoma-associated herpesvirus, KSHV) is a new herpes virus isolated from patients with AIDS-associated Kaposi's sarcoma (AIDS-KS). The ORF59 protein of HHV-8 has recently been shown to encode a processivity factor (PF-8) for HHV-8-encoded DNA polymerase. By immunoscreening a cDNA library derived from the HHV-8-infected cell line TY-1, ORF59 antigen was identified in AIDS-KS patients. Immunoblotting revealed that recombinant ORF59 protein reacted with sera from patients with AIDS-KS. Enzyme-linked immunosorbent assay (ELISA) using ORF59-recombinant protein as the antigen revealed that 7 of 22 (31.8%) AIDS-KS patients and 6 of 263 (2.2%) Japanese HIV-negative patients or healthy blood donors were positive for anti-ORF59 antibodies. Immunohistochemistry using anti-ORF59 rabbit antibodies revealed that this protein was expressed in some of the tumor cells found in KS tissues and that ORF59 protein was detected in 11 of 22 (50%) AIDS-KS tissues. In situ hybridization indicated that some of KS tumor cells were positive for HHV-8 T1.1 mRNA in the same specimen. These data suggest that ORF59 is one of the HHV-8 encoded antigens in patients with AIDS-KS and also indicated that viral replication occurred in some of KS tumor cells. ***J. Med. Virol. 59:346–355, 1999.***

KEY WORDS: HHV-8; Kaposi's sarcoma, ORF59; antigenicity

INTRODUCTION

The newly identified human herpesvirus-8 (HHV-8; Kaposi's sarcoma-associated herpesvirus, KSHV) has been demonstrated in more than 95% of Kaposi's sarcoma lesions, regardless of HIV-infection and clinical subtype [Chang et al., 1994; Dupin et al., 1995; Huang et al., 1995; Moore and Chang, 1995; Staskus et al., 1997]. HHV-8 has also been identified in primary effusion lymphomas (PEL; body-cavity-based lymphomas, BCBL) and in several cell lines established from PEL [Arvanitakis et al., 1996; Carbone et al., 1997, 1998; Cesarman et al., 1995; Gaidano et al., 1996; Katano et al., 1999; Nador et al., 1996; Renne et al., 1996; Said et al., 1996]. These cell lines have been used in immunofluorescence assay (IFA) and Western blot assays to demonstrate antibodies to HHV-8 in patients' sera [Gao et al., 1996; Kedes et al., 1997; Rainbow et al., 1997; Simpson et al., 1996].

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The seroprevalence of antibodies to HHV-8 has been found to vary between studies, depending on the type of assay used and the countries where the investigations were carried out [Andre et al., 1997; Chatlynne et al., 1998; Davis et al., 1997; Kedes et al., 1997; Mayama et al., 1998; Melbye et al., 1998; Rabkin et al., 1998; Regamey et al., 1998; Simpson et al., 1996]. Infection with HHV-8 seems uncommon among blood donors from the USA and Northern Europe but is more common in some Mediterranean countries, including Italy and Greece, and appears widespread in Africa [Mayama et al., 1998]. In non-endemic countries like the USA and Northern Europe, sexual transmission appears to play an important role, at least among homosexual men.

To date, two methods (IFA and enzyme-linked immunosorbent assay, ELISA) have been used to detect antibodies to HHV-8. However, the target antigens differ between these methods. In IFA, latency-associated nuclear antigen (LANA), corresponding to ORF73 protein (latent nuclear antigen, LNA) in immunoblotting analysis, is the major antigen, while in ELISA, ORF65 (minor capsid protein), ORF26 (another possible minor capsid protein), or a lysate of whole viral particles have been used as antigens [Chatlynne et al., 1998; Davis et al., 1997; Pau et al., 1998; Rabkin et al., 1998; Regamey et al., 1998; Simpson et al., 1996]. The use of these different antigens seems to have resulted in discrepancy in the data reported to date [Rabkin et al., 1998].

HHV-8 DNA is known to encode more than 80 complete open reading frames (ORFs) [Russo et al., 1996]. Sera from HHV-8-infected individuals are known to react with several proteins encoded by HHV-8 ORFs. By immunoscreening a cDNA library from 12-O-tetradecanoylphorbol β-acetate (TPA)-induced BCBL-1 cells with an HIV+/KS+ serum, Chandran et al. (1998) identified 12 ORFs, ORF6, 8, 9, 25, 26, 39, 59, 65, 68, 73, K8.1A, and K8.1B, as the possible immunogens [Chandran et al., 1998]. However, no information was provided in this report as to which was the predominant antigen.

ORF 59 was cloned as a HHV-8-encoded protein reacting with a monoclonal antibody 11D1 [Chan et al., 1998]. This protein is a 50 kDa polypeptide associated with the lytic cycle of HHV-8-infected cells. A 2.3 kb mRNA is suggested to encode the ORF59 protein, together with ORF58. Lin K et al. suggested that the ORF59 protein may be a processivity factor (PF-8) for viral DNA polymerase (Pol-8), which is encoded by HHV-8 ORF9 [Lin et al., 1998].

Several in situ hybridization (ISH) studies have demonstrated HHV-8 viral mRNA in the lesions of KS.

In this study, immunoscreening of a cDNA library derived from a HHV-8-infected cell line (TY-1) was performed to identify the antigens recognized by sera from HHV-8-infected patients with KS. Two cDNAs, encoding ORF59 and 58, and ORF59, 58, and K11, were cloned and the antigenicity and expression of the ORF59 protein were investigated.

MATERIALS AND METHODS

Cell Line

Establishment and characterization of the PEL cell line, TY-1, were carried out as described previously [Katano et al., 1999]. Briefly, lymphoma cells were collected from the pericardial effusion of an AIDS patient suffering from PEL and grown in RPMI1640 culture medium supplemented with 20% fetal bovine serum, 10 ng/ml insulin (Sigma Chemical Co., St. Louis, MO), 10 ng/ml transferrin (Sigma Chemical Co., St. Louis, MO), and 10% pericardial effusion supernatant, which was obtained from the same patient. The amount of pericardial effusion supernatant was reduced gradually during passages and the cells were finally grown without effusion. Single cell cloning was performed and the cell line obtained was named TY-1. TY-1 was shown to be HHV-8-positive, EBV-negative.

Construction and Immunoscreening of cDNA Library

A cDNA phage library of unstimulated TY-1 cells was constructed in the λZAP express vector (Stratagene, La Jolla, CA) according to the manufacturer's instructions and then amplified. Approximately 10^6 phages were screened on nitrocellulose filters with serum obtained from an AIDS-KS patient with aggressive cutaneous lesions. The serum was diluted 1:200 in 1 × Block Ace (Yukijirushi, Tokyo, Japan) and incubated with filters for 1 hour at room temperature. Filters were washed in phosphate-buffered saline (PBS)-0.1% Tween 20, reacted with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG, 1:2,000; Tago Immunologicals, Camarillo, CA) and visualized with nitroblue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolylphosphate (BCIP, Promega, Madison, WI). Positive phages were selected, screened twice by the same protocol, and then examined for their reactivity with sera from patients and healthy controls. After conversion of positive phage into a phagemid by the rapid excision system (Stratagene, La Jolla, CA), and helper phage according to the manufacturer's instructions, the inserts were sequenced with a Hitachi Autosequencer (Hitachi, Tokyo, Japan) with the use of internal sequencing primers.

Synthesis and Purification of Recombinant HHV-8 ORF59 Protein

A fragment corresponding to amino acids 151-397 of the published ORF59 sequence was cloned into the *BamH*I and *EcoR*I sites of the bacterial expression vector pGEX5X-2 (Pharmacia, Uppsala, Sweden) using the PCR primers 5′-ctc gga tcc tac ctt tcc acg atc gga t-3′ and 5′-ctc gaa ttc tca aat cag ggg gtt aaa tg-3′ (the cloning sites are underlined) [Russo et al., 1996]. The resulting expression construct formed a fusion protein with glutathione-S transferase (GST) and amino acid 247 of ORF59 to produce GST- ORF59 protein. This was then expressed in *Escherichia coli* and affinity-purified using glutathione-Sepharose as described pre-

viously [Smith and Johnson, 1988]. The purity and concentration of the eluted proteins were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Production of Anti-ORF59 Antibodies

Purified GST-ORF59 protein was used to immunize rabbits to produce anti-ORF59 polyclonal antibodies. Immune sera were collected from the rabbits, partially purified twice by ammonium sulfate precipitation, and the precipitates were dissolved in PBS. The solute was then pre-cleaned by passage through a GST-Sepharose column to absorb the anti-GST antibodies, and affinity-purified with antigen columns on which GST-ORF59 protein was coupled to activated-CH Sepharose (Pharmacia, Uppsala, Sweden). The purified antibody, designated anti-ORF59, was used in the following experiments.

IFA for LANA and Western Blotting

The IFA for HHV-8 LANA was carried out as described previously [Gao et al., 1996; Simpson et al., 1996]. Patients' sera were diluted 1:40 in PBS-2% fetal calf serum. Rabbit anti-human IgG-fluorescein isothiocyanate conjugate (Dako, Copenhagen, Denmark) was used as the secondary antibody. The Western blot assay was carried out using sera from an AIDS-KS patient, from a HIV-negative Japanese blood donor, and from an EBV-positive patient with infectious mononucleosis. TY-1, BCBL-1 (HHV-8 positive, EBV-negative cell line), Raji (HHV-8 negative, EBV-positive cell line), Molt-4 (HHV-8 negative, EBV-negative cell line), GST, and GST-ORF59 fusion protein were submitted for Western blotting. To avoid non-specific reactions with GST, GST protein was added to the dilution buffer at a concentration of 100 μg/ml.

Immunohistology

To investigate the expression of ORF59 in KS tissue, skin biopsies from patients with KS were fixed in 10% formalin and embedded in paraffin. Sections (4 μm) were cut and the paraffin was removed by sequential immersion in xylene and ethanol. Sections were then treated for 15 minutes in a microwave oven (high-power setting) for antigen retrieval. Endogenous peroxidase was blocked by immersing the sections in methanol-3% H_2O_2. Affinity-purified antibodies to ORF59, diluted 1:1,000 in PBS-5% bovine serum albumin (BSA), were then added and allowed to react for 1 hour at room temperature. Following two washes in PBS, biotin-conjugated anti-human IgG (Dako, Copenhagen, Denmark), diluted l:500 in PBS-5% BSA, was applied for 30 minutes. This was followed by treatment with streptavidin-conjugated horseradish peroxidase (Dako, Copenhagen, Denmark), diluted according to the manufacturer's instructions, and visualized with diaminobenzidine (0.3 mg/ml)-1% H_2O_2 for 10 minutes.

In Situ Hybridization (ISH)

ISH was performed to detect HHV-8-T1.1/*nut-1*. The digoxygenin (DIG)-labeled 50 bases oligonucleotide, 5′-gag cgc tcc cag ctg ccg cac acc act tta gtc caa tgt tct tac act ac-3′, was used as a probe [Zhong et al., 1996]. The DIG Oligonucleotide 3′-Tailing Kit (Boehringer Mannheim, Mannheim, Germany) was used for the labeling the probe. Deparaffined sections were digested with proteinase K (10 μg/ml) at 37°C for 15 minutes and rinsed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2. The slides were treated with 0.2 M HCl for 10 minutes, rinsed with PB, and dehydrated with a graded series of ethanol. Hybridization was performed in 20 μl of hybridization mixture per slide, consisting of 30% formamide, 2× SSC (0.15 M NaCl, 0.015 M sodium citrate), 1× Denhardt's solution [0.02% bovine serum albumin (BSA) fraction V, 0.02% Ficoll type 400, and 0.02% polyvinylpyrrolidone], 150 μg/ml of single stranded herring sperm DNA, 10% dextran sulfate, and 10 pmol/ml of the DIG-labeled oligonucleotide probe. After hybridization for 15 hours at 37°C, the slides were rinsed at room temperature in 2× SSC for 10 min, twice for 10 min in 0.5× SSC, and three times for 5 minutes in DIG buffer 1. After blocking with DIG buffer 2 for 30 minutes, the slide were rinsed three times for 3 minutes with DIG buffer 1 and incubated with anti-DIG antibody, diluted 1:500, for 30 minutes at 37°C. Detection was performed with NBT and BCIP.

Serum Samples

Twenty-two sera from HIV+/KS+ adult male patients, 57 sera from HIV+/KS– patients (including 51 homosexual or heterosexual transmitted and 6 hemophiliac patients), and 263 sera from HIV–/KS– individuals, composed of patients with various diseases including 59 child patients (1–15 years old) and 22 healthy donors were used in this study. To investigate the cross reaction with EBV, we tested 13 sera from patients with infectious mononucleosis, whose sera were confirmed to be positive for anti-EBNA (EB virus nuclear antigen) or anti-VCA (viral capsid antigens) by ELISA. All sera were stored at –20°C and heat-inactivated at 56°C for 30 minutes before use.

Development of ELISA Using Recombinant HHV-8 ORF59

Purified recombinant GST-ORF59 (2 μg/ml) was coated onto wells of ELISA plates (50 μl/well; EIA/RIA plate, Corning Coaster 3690, NY). Sera or plasma at various dilutions were allowed to react for 30 minutes with the recombinant protein. To avoid non-specific reactions with GST, recombinant GST protein was added to the dilution buffer at a concentration of 100 μg/ml beforehand. Unbound serum was then removed with washing buffer containing Tween-20. Goat anti-human Immunoglobulines (Igs)-alkaline-phosphatase conjugate (Tago Immunologicals, Burlingame, CA) was then added, and, after washing, phosphate substrate tablets

TABLE I. Comparison of ORF59-ELISA, IFA, and Western Blotting*

			ELISA titer (OD_{405})							IFA titer[b]		Western blot	
Number	HIV	KS	×20	×40	×80	×160	×320	×640	Result[a]	LANA	lytic	TY-1[c]	GST-ORF59[d]
1	+	+	0.329	0.244	0.166	0.131	0.091	0.023	40	>640	>640	–	–
2	+	+	0.601	0.315	0.231	0.135	0.094	0.022	80	–	>640	–	–
3	+	+	0.540	0.338	0.257	0.154	0.124	0.030	80	–	–	–	+/–
4	+	+	1.899	1.684	1.183	0.768	0.481	0.278	>640	>640	320	+	+
5	+	+	1.939	1.830	1.705	1.070	0.689	0.384	>640	>640	>640	+	+
6	+	+	1.320	0.698	0.451	0.212	0.143	0.066	80	>640	20	–	–
7	+	–	0.812	0.722	0.389	0.270	0.164	0.092	160	–	–	–	–
8	+	–	1.793	1.739	1.273	0.638	0.380	0.171	320	–	–	+	+
9	+	–	1.821	1.797	1.505	1.099	0.634	0.351	>640	–	>640	+	+
10	+	–	0.262	0.181	0.097	0.089	0.070	0.016	20	–	–	–	–
11	–	–	0.200	0.134	0.100	0.065	0.055	0.001	–	–	–	–	–
12	–	–	0.256	0.158	0.179	0.126	0.118	0.005	20	–	–	–	–
13	–	–	0.259	0.160	0.107	0.074	0.068	0.017	20	–	–	–	–
14	–	–	0.168	0.080	0.042	0.024	0.034	–0.003	–	–	–	–	–
15	–	–	0.081	0.040	0.020	0.011	0.067	–0.003	–	–	–	–	–
16	–	–	0.352	0.214	0.118	0.065	0.063	0.006	20	–	–	–	–
17	–	–	0.142	0.128	0.078	0.045	0.067	–0.001	–	–	–	–	–
18	–	–	0.132	0.060	0.016	0.017	0.040	–0.005	–	–	–	–	–
19	–	–	0.034	0.017	0.009	0.003	0.023	–0.008	–	–	–	–	–
20	–	–	0.088	0.041	0.026	0.015	0.038	–0.003	–	–	–	–	–

*Ten HIV + patients (No. 1–10), including six AIDS-KS patients, and ten healthy donors (No. 11–20) were tested by ELISA for ORF59, and IFA for LANA and nuclear antigens and were used in Western blotting of TY-1 cell lysates and GST-ORF59 fusion proteins. Sera were titrated from 1:20–1:640 in the ELISA and IFA. A cut-off value of 0.227 was chosen for the ELISA from the results with HIV–/KS– patients/donors sera. "LANA" in the IFA indicates dot-like signals in the nucleus, whereas "lytic" indicates diffuse nuclear and/or cytoplasmic staining. In Western blotting, all sera were diluted 1:100.
[a]Cut off value = 0.227.
[b]Positivity of latent protein (LANA) and lytic protein (other proteins) in IFA.
[c]Positivity of 50kDa protein in cell lysates of TY-1 (HHV-8 infected cell line).
[d]Positivity of recombinant GST-ORF59 fusion protein (70 kDa).

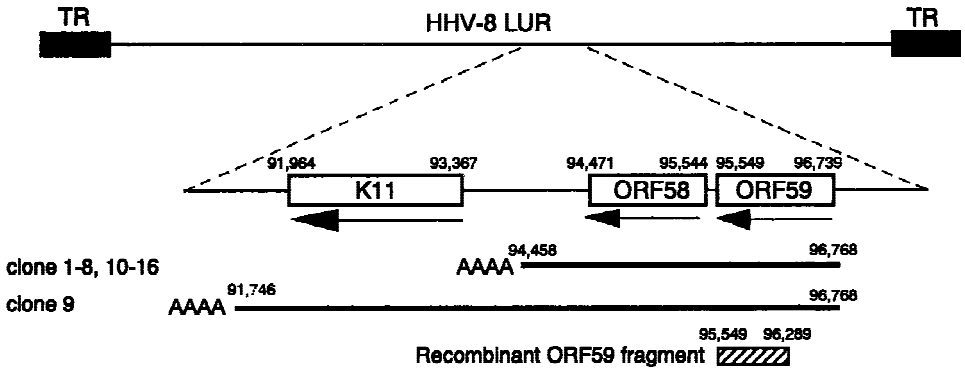

Fig. 1. Mapping of cDNA clones obtained by immunoscreening. The positions of the ORFs in the non-conserved genomic region of HHV-8 are indicated by open boxes, and the nucleotide numbers marking their beginning and end are listed in the published orientation of the genome [Russo et al., 1996]. The coordinates of the corrected ORF59/ORF58/K11 sequence are shown [Chan et al., 1998; Russo et al., 1996]. Arrows indicate the direction of transcription. Lines indicate the locations of two distinct cDNA clones (clones 1–8, 10–6, and 9). The position of the recombinant ORF59 fragment used to affinity-purify ORF59-specific antibodies is also shown.

(5 mg/tab, Sigma Chemical Co., St. Louis, MO) were used to develop the color. The absorbance of the wells was measured at 405 nm.

Based on our negative control sera and surveys of patients with KS, an ELISA titer of 1:100 was considered appropriate for reaction. This dilution was chosen to evaluate the results of the ELISA, taking into consideration the results of IFA on these sera (see Results and Table I). The cut-off value for the ELISA was chosen as follows: negative control samples (263 HIV–/KS– patients or healthy donors) with an absorbance above the mean + 1 SD (standard deviation) were excluded, and the mean absorbance + 3 SD was chosen as the cut-off value for the remaining samples. Thus, the cut-off value was calculated as 0.227 (OD405).

RESULTS

Immunoscreening and Mapping of cDNA clones

TY-1 is a PEL-derived cell line infected with HHV-8 but not with EBV [Katano et al., 1999]. A large proportion of TY-1 cells are infected latently with HHV-8, as shown by weak expression of the lytic gene T1.1/*nut-1* in Northern blotting, and augmentation of this expression by treatment with phorbol ester [Katano et al., 1999; Zhong et al., 1996]. When clones (10^6) of a cDNA

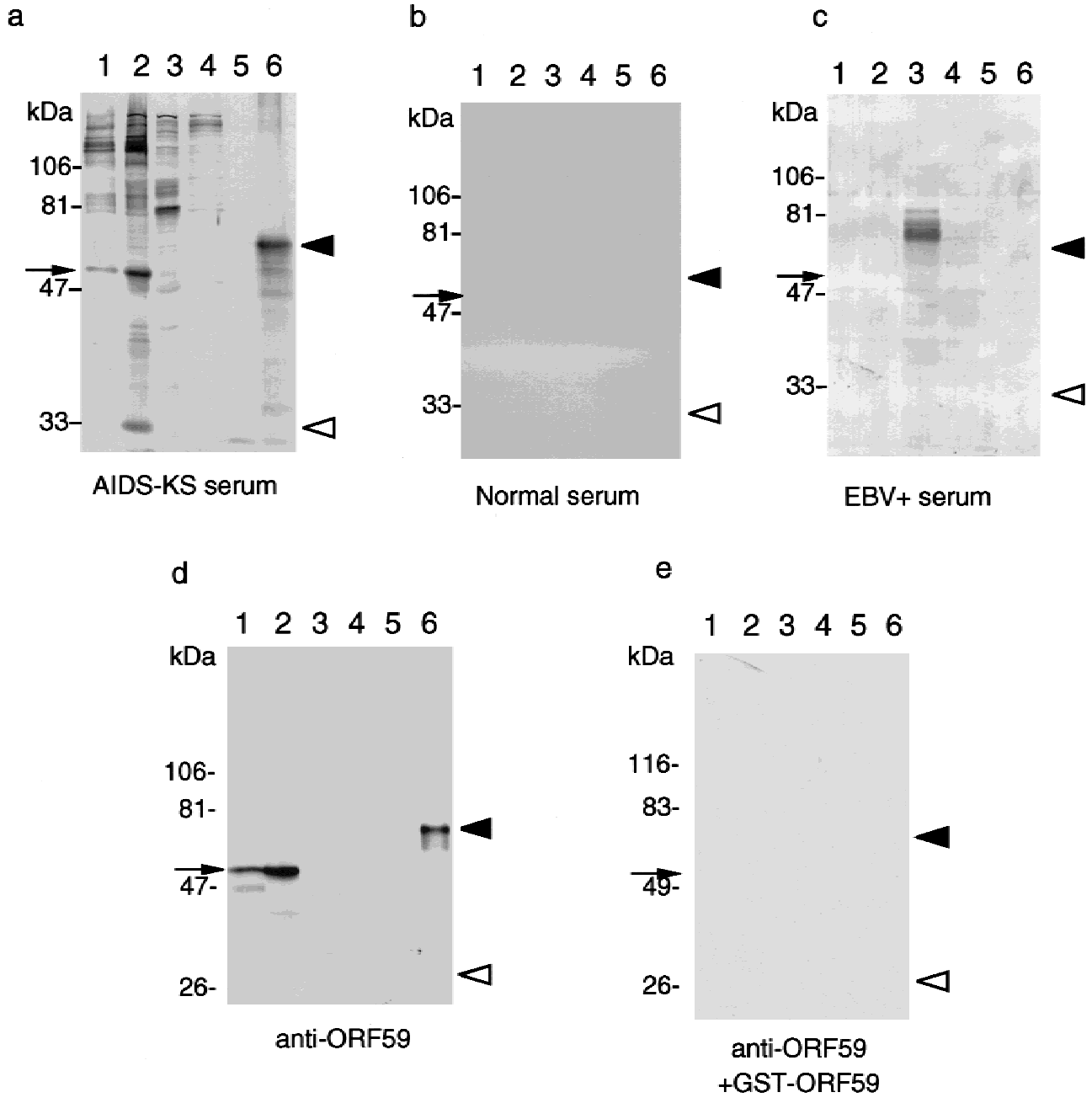

Fig. 2. Reaction of AIDS-KS serum with ORF59. Lane 1: BCBL-1 (HHV-8-positive, EBV-negative cell line), lane 2: TY-1 (HHV-8-positive, EBV-negative cell line), lane 3: Raji (HHV-8-negative, EBV-positive B-cell line), lane 4: Molt-4 (HHV-8-negative, EBV-negative T-cell line), lane 5: GST protein (26 kDa, open arrowhead), lane 6: recombinant GST-ORF59 protein (approximately 70 kDa, closed arrowhead). **a:** AIDS-KS serum was used in Western blot analysis with various cell lines, GST and GST-ORF59. The AIDS-KS serum recognized the Western blot-defined 50 kDa protein in two HHV-8-infected PEL cell lines, BCBL-1 and TY-1 (arrow) but not in two HHV-8-negative cell lines, Raji and Molt-4. The AIDS-KS serum also reacted with some proteins (230, 220, 180, 150, 85, and 50 kDa) in BCBL-1 and TY-1, and with the 75 kDa protein (presumably EBNA1) in Raji [Gao et al., 1996]. Recombinant GST-ORF59 was stained with the AIDS-KS serum, while GST protein was not stained. **b:** Normal serum did not react with any of the proteins in the cell lysates or with recombinant proteins. **c:** EBV-positive serum from a patient with infectious mononucleosis did not react with GST-ORF59. No bands were found in BCBL-1, TY-1, and Molt-4, while EBV-positive serum reacted with a 75 kDa protein in the lysate of Raji cells. **d:** Affinity-purified rabbit antibodies to ORF59 recognized the Western blot-defined 50 kDa protein. A 50 kDa band was detected in the cell lysates from two HHV-8-infected cell lines (BCBL-1 and TY-1) but not in two HHV-8-negative cell lines (Raji and Molt-4). This 50 kDa band corresponded to the band in (a). **e:** Recombinant GST-ORF59 protein blocked the reaction of anti-ORF59 rabbit antibodies with the 50 kDa protein and with recombinant GST-ORF59 protein completely.

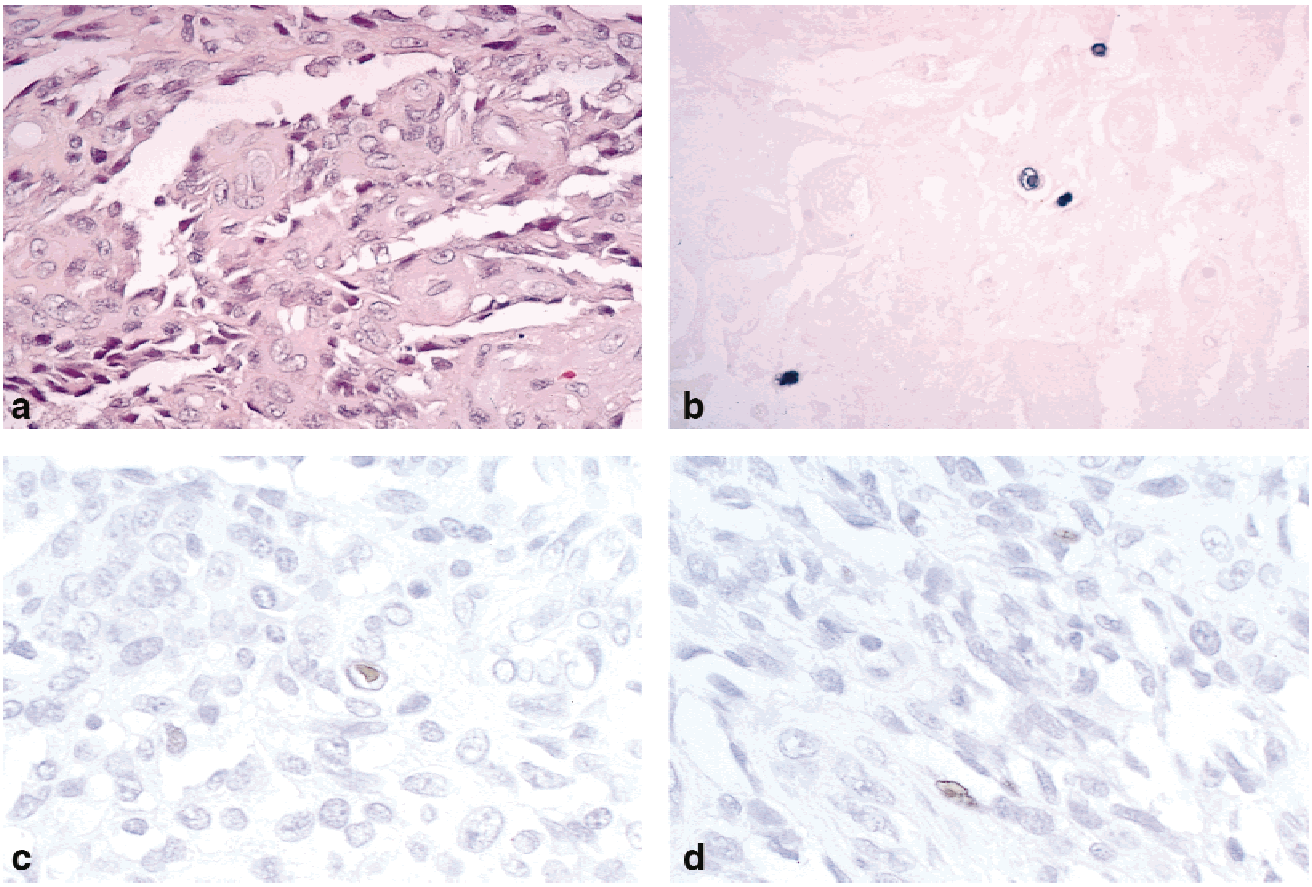

Fig. 3. ORF59 is expressed in KS tumor cells. **a:** Hematoxylin and eosin staining of a biopsy sample of nodular KS tissue. **b:** ISH using the DIG-labeled oligonucleotide probe to HHV-8-encoded T1.1 mRNA. **c,d:** Stained with affinity-purified rabbit antibodies to ORF59. A small proportion of KS tumor cells expressed ORF59 in their nuclei. In some of cells, ORF59 was expressed in intranuclear inclusion body-like structures. The staining pattern of ORF59 was similar to that of ISH for T1.1.

library constructed from uninduced TY-1 cells were screened using serum from a patient with AIDS-KS, 16 positive clones were obtained (Fig. 1). Sequence analysis revealed that these clones consisted of two groups (Fig. 1): the first (clones 1–8, 10–16) is a 2,310 bp cDNA with a polyadenylation signal sequence corresponding to mRNA derived from HHV-8 ORF59 and ORF58 mRNAs [Chan et al., 1998], while the second (clone 9) is a 5,019 bp cDNA, starting at nucleotide 96,768 of the published sequence [Russo et al., 1996], which is located upstream of ORF59, and extending to the end of ORF59 and beyond through ORF58 and a downstream ORF (ORF K11). Termination occurs at nucleotide 91,750 downstream from K11, which contains an ATAAA polyadenylation site at nucleotide position 91,750–91,754. The latter mRNA apparently encodes three ORFs: ORF59, ORF58, and K11. Our cloned sequences, clones 1–8, 10–16, were matched to the published ORF59 by 99%, and we could found no constant mutation. Since ORF59 in all clones, and K11 in clone 9, were in frame with the β-galactosidase fusion partner, it was concluded that the predominant protein recognized by this AIDS-KS serum was ORF59.

AIDS-KS Sera React with the 50 kDa Protein, Corresponding to ORF59, in Western Blotting

The first ORF within the cDNA cloned by the present immunoscreening (i.e., ORF59) was contiguous with the β-galactosidase-encoding gene in the λZAP vector. This suggests that ORF59 contains an epitope that reacts with sera from patients. A 247 amino acids length fragment of ORF59 was successfully expressed in *Escherichia coli* as a fusion protein with GST (Fig. l). On Western blotting, the purified GST-ORF59 recombinant protein (approximately 70 kDa) reacted with serum from a patient with AIDS-KS, but not with normal serum or serum from an EBV-infected patient (Fig. 2a–c). The AIDS-KS serum also reacted with some proteins (230, 220, 180, 150, 85, and 50 kDa) derived from HHV-8-infected cell lines (BCBL-1 and TY-1), while normal serum did not (Fig. 2a,b). The AIDS-KS serum also reacted with bands, which were approximately 75 kDa in size (EBNA1), in the EBV-positive Raji cell line [Gao et al., 1996]. To confirm the absence of cross-reactivity between this serum and EBV-encoded proteins, the reactivity of ORF59 and lysates of these cell lines was determined using serum derived from an EBV-infected

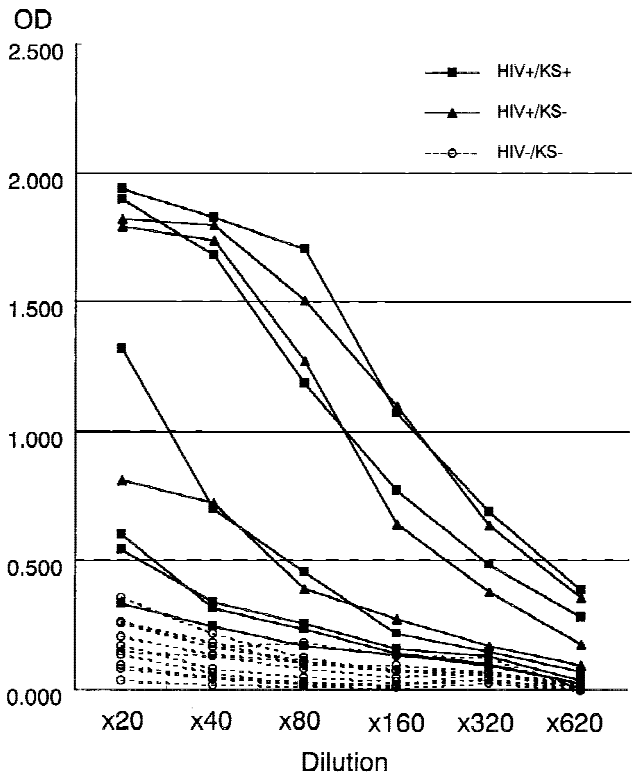

Fig. 4. ELISA anti-ORF59 antibody titers. Sera from a HIV+/KS+ patient (closed boxes with solid lines), HIV+/KS– patients (closed triangles with solid lines), and HIV–/KS– patients or healthy donors (open circles with broken lines) were blotted. Data shown are converted from Table I.

patient. This serum recognized a 75 kDa protein only in the Raji cell line but did not recognize any proteins in BCBL-1 or TY-1 (Fig. 2c).

The 50 kDa protein recognized by the AIDS-KS serum in BCBL-1 and TY-1 was speculated to be ORF59 [Chan et al., 1998]. To confirm this, ORF59-specific polyclonal rabbit antibodies were produced using the recombinant GST-ORF59 fragment as the immunogen. The affinity-purified ORF59-specific antibodies produced a dense, diffuse nuclear staining pattern on some cells (approximately 3%) of the TPA-treated HHV-8-infected cell line, TY-1 (data not shown). The antibodies also reacted with recombinant GST-ORF59 proteins and with the 50 kDa protein in BCBL-1 and TY-1 cell lysates (Fig. 2d). This antibody did not recognize any bands in HHV-8-negative cell lines, including Raji and Molt-4 (Fig. 2d). The recombinant ORF59 fragment blocked this positive reaction completely (Fig. 2e). These findings demonstrate that the 50 kDa protein in BCBL-1 and TY-1 defined by Western blotting is ORF59 protein and that the serum from the patient with AIDS-KS contained antibodies to ORF59 protein.

ORF59 Is Expressed in Tissue From KS Lesions

The expression of ORF59 in tissue from KS lesion was examined using affinity-purified rabbit antibodies to ORF59 protein. As shown in Figure 3c,d, a sparse immune reaction was noted in a small number of KS cells. Similar positive cells were identified in 11/22 tissue samples from AIDS-KS patients. These positive cases corresponded to < 10 positive cells per high power view. Nuclei were the site of the immune reaction, while structures such as inclusion bodies were identified occasionally. The same type of reaction was noted by ISH in HHV-8-infected cells in the lytic phase (Fig. 3b).

Establishment of an ELISA to Detect Anti-ORF59 Antibodies

To investigate the reactivity of human sera with ORF59, an ELISA system was established using the recombinant GST-ORF59 fusion protein.

In order to check the specificity of the ELISA, the reactivity and titer of the sera were tested simultaneously by IFA for LANA and Western blotting for HHV-8-positive cell lines (Table I, Fig. 4). Ten sera from AIDS-patients, including five with KS, and ten sera from healthy blood donors were tested. As shown in Table I, the data of ELISA and IFA matched by 16/20 (80%), and ELISA and Western blotting by 19/20 (95%). When the titers were determined, IFA was found to be more sensitive than ELISA for three sera (cases 1, 2, and 6), while two sera (cases 7 and 8) were positive only by ELISA. Western blot analysis again correlated with the ELISA results. All 13 sera from patients with infectious mononucleosis were negative in this ELISA and IFA (data not shown). Thus, it was concluded that our ELISA was specific for ORF59 protein and that the discrepancy between the ELISA and IFA was due to the different antigens that were detected by these two assays. Titration experiments showed that ODs of positive sera changed prominently in 1:80–1:160, and thus dilution of 1:80–1:160 seemed optimal for this ELISA (Fig. 4).

Seroprevalence of Antibodies to ORF59 in Japanese Subjects

The seroprevalence of anti-ORF59 antibodies among Japanese AIDS-KS, HIV-positive patients, HIV-negative patients, and healthy blood donors was investigated using the ELISA for ORF59. Seven of 22 (31.8%) sera from AIDS-KS patients, and 15 of 57 (26.3%) sera or plasma samples from HIV+/KS– patients were found to have antibodies to ORF59 (Fig. 5). Antibodies were found in six out of 263 (2.2%) HIV–/KS– sera that were composed of patients and/or healthy blood donors. All of these ELISA-positive sera reacted with latent and/or lytic antigens by IFA. Sera from four HIV+/KS+ patients gave a high absorbance (> 0.3), and these patients were subsequently shown to have involvement of KS in internal organs or multiple skin lesions. All ELISA-negative cases in HIV+/KS+ patients had a single cutaneous KS lesion and had a good prognosis in terms of the development of KS.

DISCUSSION

In the present study, HHV-8-encoded ORF59 protein was shown to be one of the antigens recognized by sera from patients with AIDS-KS, especially those with

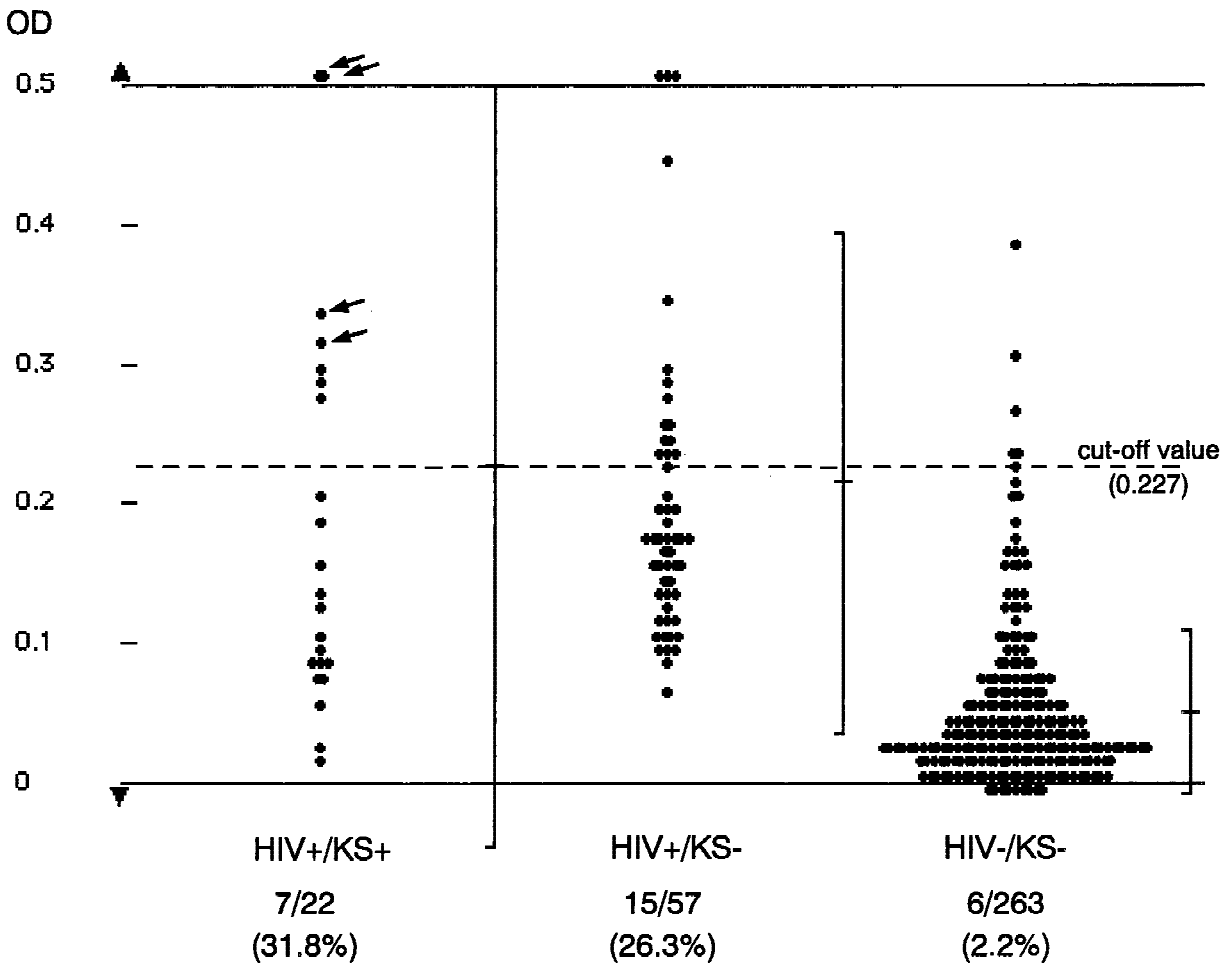

Fig. 5. Group scattering of ORF59-ELISA results. The results of the HIV+/KS+ patients, HIV+/KS– patients and HIV–/KS– patients, or healthy donors are shown. One dot represents the data for one patient. The cut-off value (0.227) is indicated by a broken line. Arrows indicate aggressive KS cases with intra-abdominal involvement or multiple lesions.

more aggressive KS. This conclusion is based on the observations that 1) all cDNAs cloned by immunoscreening using serum from a patient with AIDS-KS, encoded the ORF59 protein; 2) sera from AIDS-KS patients reacted with the recombinant GST-ORF59 fusion protein, as well as the 50 kDa ORF59 protein derived from HHV-8-infected cells when tested by immunoblotting; 3) the ORF59 protein was expressed in tissue samples taken from KS lesions; 4) 32% of AIDS-KS sera, but only 2.2% of HIV–/KS– patient sera reacted with the ORF59 protein in ELISA; and 5) HIV+/KS+ patients exhibiting a high titer of anti-ORF59 antibodies had metastatic or multiple KS lesions.

So far, there have been only three reports dealing with cDNA cloning of HHV-8 in combination with immunoscreening. Lin et al. [1997] identified ORF65 as a small viral capsid antigen (sVCA) by screening a cDNA library of the HHV-8-infected cell line BC-1. Likewise, Rainbow et al. [1997] identified ORF73 (LNA) as an HHV-8-encoded major antigen from HBL-6 cell line. Chandran et al. [1998] identified 12 cDNAs, including ORF59, from BCBL-1. However, it was surprising that all the cDNAs cloned in our study encoded the same protein, ORF59. It is probable that the differences in these results were due to the different sera used.

ORF59 is a DNA replication protein cloned from HHV-8 [Chan et al., 1998]. ORF59 (PF-8) is required by HHV-8-encoded DNA polymerase (Pol-8) to synthesize greatly extended DNA products (> 7,000 nucleotides in in vitro DNA synthesis assays), whereas Pol-8 can only incorporate several nucleotides [Lin et al., 1998]. Western blotting using anti-ORF59 antibody revealed that ORF59 protein was induced in HHV-8-infected cell lines by TPA treatment [data not shown and Chan et al., 1998]. This result showed at least that ORF59 protein was expressed more abundantly in the lytic phase than in the latent phase. These data suggest that ORF59 is a "lytic protein" and is expressed in cells before and during viral DNA replication. Our immunohistological results seem to support the idea that ORF59 is expressed as a lytic phase-associated protein. Furthermore, our data also suggest that viral replication occurs in the nucleus, as is known to be the case with other herpes viruses. Our immunohistochemistry also showed that the ORF59 protein could not be demonstrated in 50% of AIDS-KS patients. In addition,

ORF59 was demonstrated in only a small number of cells within KS tissue, even in the tissue samples which stained positively. As the pattern of immunostaining in KS tissue seems to reflect viral DNA synthesis, it appears that such viral replication occurs in a limited number of KS tumor cells. As four patients with aggressive KS exhibited high antibody titers to ORF59 by ELISA, the expression level of ORF59 as a result of viral replication may somehow be related to the progression of KS. No useful markers have been found in KS tissue that might predict progressive growth. Considering the function of ORF59 as a processivity factor, and the potent antigenicity of this protein in AIDS-KS patients, ORF59 is likely to be one of the important factors determining the prognosis of KS. In this sense, the detection of ORF59 may be useful in defining the clinical grade of KS. However, further detailed studies are needed to estimate the association between ORF59 and the progression of KS.

The specificity of our ELISA was basically confirmed by 1) correspondence of IFA with Western blot in the results, 2) the titration depending on the concentration of HIV+/KS+ sera, and 3) no reaction with sera of infectious mononucleosis patients. In our ELISA, the seroprevalence of antibodies to HHV-8 (31.4%) in AIDS-KS patients was the lowest reported to date [Andre et al., 1997; Chatlynne et al., 1998; Davis et al., 1997; Mayama et al., 1998; Melbye et al., 1998; Pau et al., 1998; Rabkin et al., 1998; Regamey et al., 1998; Simpson et al., 1996]. In addition, our ELISA was not as sensitive enough when compared with IFA for LANA. This low sensitivity may be attributed to the fact that the antigenicity of ORF59 was not so high as that of ORF65, ORF26, whole viral lysate, or LANA. Moreover, the sera we used in this study as HIV–/KS– sera included patients' sera with various diseases in addition to those of healthy people. Thus, we could not decide in this study the seroprevalence rate of HHV-8 among the Japanese general population.

There is only limited data regarding HHV-8 seroprevalence rates in the general Japanese population [0.2% by Fujii et al., 1998], whereas almost all Japanese are infected with EBV [Imai, 1990]. A recent report suggested that transmission of HHV-8 may resemble that of EBV in EBV-endemic countries [Mayama et al., 1998]. In such countries, HHV-8 appears to follow a horizontal pattern of transmission similar to most other herpes viruses, whereas in Northern Europe and the USA, HHV-8 is thought to be transmitted sexually. Further studies are, therefore, required to determine the predominant pattern of transmission of HHV-8 among the Japanese people.

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